Selection of DNA ligands for protein kinase C-δ†

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Protein kinase Cs are a family of serine and threonine kinases that mediate a wide variety of cellular signalling processes such as cell growth, differentiation, apoptosis and tumor development. We have selected high-affinity DNA aptamers for PKCδ by capillary electrophoresis based SELEX (systematic evolution of ligands by exponential enrichment, CE-SELEX). We have demonstrated that fluorescently tagged PB9 aptamer can specifically recognize PKCδ under *in vitro* conditions. The K_d of the aptamer-protein binding is 122 nM. These aptamers will enable us to apply fluorescently labelled probes to study the spatiotemporal dynamics and activation of individual endogenous PKC isoforms during various cell signalling processes.

We have selected high-affinity DNA aptamers for Protein kinase C δ (PKCδ) by capillary electrophoresis based SELEX (systematic evolution of ligands by exponential enrichment, CE-SELEX). PKCs are a family of serine and threonine kinases that mediate a wide variety of cellular signalling processes such as cell growth, differentiation, apoptosis and tumor development.[1] Among PKC isoforms, PKCδ, an important member of the novel PKC family, plays a critical role in various cellular signal transduction pathways and has been extensively studied.[2–4] It has been reported that subcellular localization of PKCs, including PKCδ, is the key to their cellular function and regulation.[5] Typically, subcellular localization of PKCs has been determined by immunostaining of fixed cells using PKC-specific antibodies[6] or by following the movement of overexpressed fluorescent-labeled PKCs in live cells. Although the latter method allows real-time monitoring and thus generally preferred over the former, several groups have reported that overexpressed PKCs may overwhelm endogenous enzymes, causing non-specific and non-physiological localization and substrate phosphorylation.[7,8] For this reason, development of a new methodology that allows specific fluorescence labeling of endogenous proteins, including PKCs, would be of great importance.

It has been demonstrated that protein–protein interactions can be studied using a fluorescently labelled aptamer.[9–11] One way to obtain such protein specific DNA aptamers is by capillary electrophoresis based SELEX (CE-SELEX).[12,13] This method has shown significant advantages over conventional SELEX techniques. For example, the first study on CE-SELEX was reported to obtain aptamers with low-nanomolar dissociation constants (K_d) in only four rounds of CE-SELEX.[11] In comparison with affinity column methods, CE-SELEX selection occurs in free solution, eliminating the unfavorable kinetics associated with elution of high-affinity sequences. In addition, this method presents much less non-specific binding of DNA to the solid support observed in filter techniques.[12,13] In this work, we used CE-SELEX to identify DNA sequences that specifically bind to PKCδ, with the objective of producing fluorescent probes for both intracellular and *in vitro* studies.

The initial DNA library consisted of a pool of oligonucleotides containing a randomized sequence of 30 nucleotides (see Fig. 1(A)), flanked on both ends by the two primer hybridization sites with sequences identical to those described by Sanchez *et al.*[14] We have optimized linear symmetric Polymerase Chain Reaction (PCR) conditions compatible with CE-SELEX (see ESI,† Fig. S1). All oligonucleotides, including PCR primers, were synthesized using standard phosphoramidite chemistry and purified by denaturing polyacrylamide gel electrophoresis to remove truncated DNA fragments produced in the synthesis.

The initial libraries used in the first SELEX round typically contain 10^{13} to 10^{15} independent sequences.[15] In comparison, even with higher than usual injection volumes, this number is significantly less for CE-SELEX than conventional SELEX techniques due to the very low sample capacity of the capillary. Therefore, we initiated the SELEX process by using 2 mM DNA

A

5'-GCCAGGGGTTCCACTACGTAGA-N_{30}-ACCAGGGGGCAGAGAGAAGGGC-3'

Reverse primer
3-CGGTCCCCAAGGTTGAGCATCA

B.

	Sequence	Percentage of Population (%)
PB 9	ACACGACGGGAATACT-GACTCTCCCCCATGT	32
PB 12	TGGTGAACGGAATGCCGGGGCTTCCACTAC GCAGA	4
PB 11	CATGCTGCCAGGGGTTCCACTACGTAGAGGCAA	24
PB 2	CCAGGGGGCAGAGAGAAGGGCATGGTGTG-	11
PB 1	GTAAAGGGCCAAAGACTGTATGAATACCAT	9
PB 3	AGCCGAGTGCTCGCAACGGTTTAGCCCCAT	4
PB 4	CAACGAGAGTAGAACGAGGGGATGTCTGCA	4
PB 6	GGACGGGCAAAGAAAGAGGGAAGAGAACAG	4
PB 5	GCCAAGAGCAACGAGGAAGCAGGATAGGGC	3
PB 7	CATACGTGGTCATGCATACCCGTAACCGTT	2
PB 8	ACAAAAGAAGGAGAGGGAGAAGGGATAGGT	2

Fig. 1 SELEX library and DNA sequences. (A) Randomized library and primers used for the selection. (B) Evolved sequences without the primer sites obtained after round 9 with percentage of population.

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library, which would generate 10^{13} DNA molecules with an injection volume of a few nanoliters.

An electropherogram was generated using 2 mM library, shown in Fig. S2 in ESI.† Throughout the subsequent SELEX rounds, the initial DNA library was denatured and then slowly cooled at room temperature to allow the formation of stable secondary structures before incubating with PKCδ. During each round, PKCδ was kept at 100 nM concentration and incubated with the DNA library at room temperature for 15 min to allow complete binding. Separation and collection of DNAs bound to the targets were performed on a homemade CE system which was described elsewhere.[16] A poly(vinyl alcohol) coated capillary (Agilent Technologies, Palo Alto, CA) with 50-μm inner diameter, 360-μm outer diameter, and a length of 35 cm (25 cm to the detector window) was used. The polymer coating helps to reduce adsorption of proteins on to the capillary wall.[16] A potential of 12.5 kV was applied during separations up to four rounds of selection. After the fourth round, the running voltage was decreased to 7.5 kV to prevent base line interferences due to Joule heating. By reducing the heating-induced noise, detection of a lower concentration of DNA was made possible in the later rounds of selections. All samples were injected into the capillary hydrodynamically (Δh = 5 cm, 10 s), and absorbance at 254 nm of free DNA was monitored using a UV/Vis detector (CE-Thermo Capillary Electrophoresis, CRYSTAL 110). In most rounds, more than one CE fractions of DNA bound PKCδ were captured to increase the efficiency of the selection. Collected fractions were PCR amplified with 50 μL reactions volumes. A second PCR step was employed with a biotinylated reverse primer. The amplified products were converted to single stranded DNAs using a streptavidin column.

As the free DNA sequences should theoretically migrate with the same mobility in CE due to similar charge-to-mass ratios, the formation of a complex with the target protein at its pI alters the mobility of the DNA, causing bound sequences to migrate at a slower rate. Therefore, DNA fractions bound to PKCδ were collected following the elution of unbound sequences off of the capillary.

The progress of the selection was monitored and investigated by Electro Mobility Shift Assay (EMSA) using ^{32}P labelled DNA aliquots from each pool. By comparing the binding curves corresponding to the initial library (ESI,† Fig. S3) and enriched pool from round 9 (see Fig. 2), we observed a considerable enrichment of high-affinity sequences.

Finally, in order to capture the sequences with the highest affinity, we introduced an additional gel extraction step. An EMSA assay was performed using 8 nM of PKCδ with an excess amount of DNA to increase the competition which favoured binding of high-affinity aptamers to the limited protein molecules. DNA sequences bound to protein were extracted using the crush and soak method where the band corresponded to DNA–protein complex was extracted from the gel, crushed, and soaked in the elution buffer to elute bound DNA. The DNA bound to the protein was recovered by phenol extraction followed by ethanol precipitation.

Eluted fractions from the gel extraction were PCR amplified, cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), ligated into the TA cloning vector, and transformed into *Escherichia coli*. White colonies were isolated and sequenced using a 96-well format MegaBACE 1000 capillary sequencer (GE Healthcare). The resulting clones were analyzed with the software Sequencing Analysis Clustal W 6.0. The sequenced clones gave 86 sequences, grouped into 11 sub-families based on sequence similarity as shown in Fig. 1(B).

Binding studies showed that, out of the 11 sequences, sequence PB9, with the highest population, showed the uppermost affinity in the initial EMSA experiments (see ESI,† Fig. S4). Specificity studies of PB9 were assessed by measuring its affinities for PKCδ and closely related PKCα and PKCθ. As shown in Fig. 3 the specificity of PB 9 towards PKCδ is significantly higher than those of the other two isoforms. This is significant because PKCδ and PKCθ have high-sequence homology.

We have then labeled clone PB9 with TMR (tetramethylrhodamine) at the 5′ end. The binding affinity of the modified PB9 was investigated using fluorescence anisotropy.[17] Fig. 4 shows the binding curve, which was generated by monitoring the change in fluorescence anisotropy of PB9 upon addition of PKCδ. The dissociation constant (K_d) calculated for PB9 was 122 nM. A control experiment was carried out using a TMR labeled random DNA sequence. It showed no significant change of fluorescent

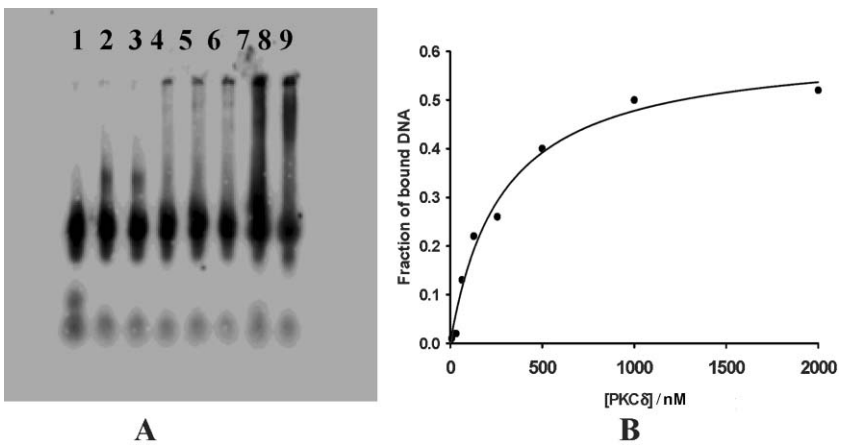

Fig. 2 (A) EMSA image (4% PAGE) observed for labelled enriched pool from round 9. Binding of the aptamer to PKCδ is significantly increased. (B) Fraction bound plotted as a function of protein concentration. Lanes 1–9: 3 nM DNA with 4, 8, 16, 32, 64, 128, 250, 500, 1000, 2000 nM of PKCδ.

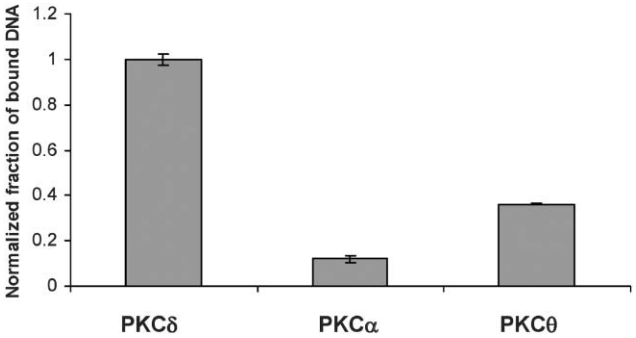

Fig. 3 Specificity of PB9 for PKCδ. 25 nM of PB9 bound to fixed concentrations of PKCδ, PKCα and PKCθ, respectively, are normalized and compared.

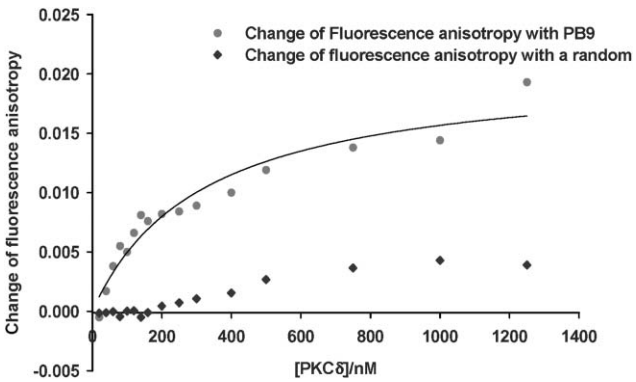

Fig. 4 Anisotropy changes of 5′-TMR labelled PB9 upon addition of PKCδ

anisotropy when PKCδ was added. This demonstrates that the fluorescently labeled probe PB9 can bind with PKCδ specifically with high affinity.

In conclusion, we have selected DNA aptamers capable of *in vitro* PKCδ monitoring using CE-SELEX. We have demonstrated that fluorescently tagged PB9 can specifically recognize PKCδ under *in vitro* conditions. The K_d of the aptamer–protein binding is 122 nM. These aptamers will enable us to apply fluorescently labelled probes to study the spatiotemporal dynamics and activation of individual endogenous PKC isoforms during various cell signalling processes. Our work presents an example of using CE-SELEX for the development of molecular probes for studying intracellular proteins using easily obtainable aptamers.[18]

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